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Exposure to Ambient Particulate Matter during Specific Gestational Periods Produces Adverse Obstetric Consequences in Mice

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Background: Epidemiological studies associate inhalation of fine-sized particulate matter ($PM_{2.5}$) during pregnancy with preterm birth (PTB) and low birth weight (LBW) but disagree over which time frames are most sensitive, or if effects are cumulative.

Objectives: Our objective was to provide experimental plausibility for epidemiological observations by testing the hypothesis that exposure to $PM_{2.5}$ during discrete periods of pregnancy results in PTB and LBW.

Methods: For the first study, timed-pregnant $B6C3F_1$ mice were exposed to concentrated ambient $PM_{2.5}$ (CAPs) or filtered air (FA) throughout pregnancy [6 h/d from gestational day (GD) 0.5 through GD16.5]. A follow-up study examined the effects of CAPs exposure during discrete gestational periods (1: GD0.5–5.5; 2: GD6.5–14.5; 3: GD14.5–16.5; 4: GD0.5–16.5) aligning to milestones during human development.

Results: In the first experiment, exposure to 160 $\mu g\,CAPs/m^3$ throughout pregnancy decreased gestational term by 0.5 d ($\sim$1.1 wk decrease for humans) and birth weight by 11.4% compared with FA. The follow-up experiment investigated timing of CAPs exposure (mean concentrations at 178, 193, 171, and 173 $\mu g/m^3$ for periods 1–4, respectively). Pregnancy was significantly shortened (vs. FA) by $\sim$0.4d when exposure occurred during gestational periods 2 and 4, and by $\sim$0.5d if exposure occurred during period 3. Exposure during periods 1, 2, and 4 reduced birth weight by $\sim$10% compared with FA, and placental weight was reduced ($\sim$8%) on GD17.5 if exposure occurred only during period 3.

Conclusions: Adverse $PM_{2.5}$-induced outcomes such as PTB and LBW are dependent upon the periods of maternal exposure. The results of these experimental studies could contribute significantly to air pollution policy decisions in the future. https://doi.org/10.1289/EHP1029

Introduction

In the United States, $\sim$11% of all pregnancies result in preterm birth (PTB; birth prior to 37 wk gestation) (March of Dimes 2014). Although the reasons for this outcome are varied, exposure of pregnant women to elevated levels of fine-sized ambient particulate matter ($PM_{2.5}$) has been identified in numerous epidemiologic studies as a contributing factor (Bell et al. 2010; Ha et al. 2014; Pereira et al. 2014; Ritz et al. 2007). Exposure to $PM_{2.5}$ is not only associated with PTB but also with low birth weight (LBW; <2,500 g) as a result of restricted fetal growth in infants born early and in those carried to full term (Ha et al. 2014). The link between $PM_{2.5}$ and increased risk for PTB was first reported by Xu et al. (1995) in a community-based cohort study. Since that time, epidemiological evidence strengthening the association between PM exposure and PTB and LBW continues to accumulate (Ha et al. 2014; Huynh et al. 2006; Jiang et al. 2007; Malmqvist et al. 2011; Ritz et al. 2000, 2007; Srám et al. 2005; Zhao et al. 2011). Such outcomes are also associated with increased risk for long-term health issues including eye/vision problems (O'Connor and Fielder 2007), learning disabilities (Johnson and Breslau 2000), and later-life chronic diseases including cardiovascular disease (Lewandowski et al. 2013) and type 1 and type 2 diabetes (Li et al. 2014).

A question that remains highly debated among human studies is whether timing of $PM_{2.5}$ exposure during pregnancy is a relevant risk factor for PTB and/or LBW. Although a number of epidemiological studies have attempted to address this critical question, the data remain inconsistent. A case–control survey performed in 2003 and nested within a birth cohort (2,543 of 6,374 women sampled in California from a cohort of $\sim$58,000 births in Los Angeles County), Ritz et al. (2007) demonstrated that the occurrence of PTB is proportional to $PM_{2.5}$ exposure levels during the first trimester only. A more recent epidemiological study by Pereira et al. (2014) reported that exposure of Hispanic women to $PM_{2.5}$ during either the first trimester or throughout the entirety of pregnancy resulted in a greater risk for PTB than at other times during pregnancy. A study from Florida revealed that maternal exposure to $PM_{2.5}$ during any point of pregnancy increased the risk for both PTB and LBW but that the second trimester was most sensitive (Ha et al. 2014). Bell et al. (2010) reported an increased risk for LBW following maternal exposure to $PM_{2.5}$ derived from oil burning, but only during the third trimester. Thus, the period (or periods) of greatest sensitivity during pregnancy for PM-induced effects on gestational duration and birth weight remains unsolved.

Studies performed using animal models to examine the effects of prenatal exposure to ambient PM on fetal or gestational outcomes are limited. A study by Veras et al. (2008) demonstrated that a 24 h/d exposure of pregnant Balb/c mice to 27.5 $\mu g/m^3$ $PM_{2.5}$ from the start of pregnancy through gestational day (GD) 17 decreased placental weight. This change in placental weight was associated with decreased blood vessel diameter on the maternal side of the placental vasculature; capillary surface area on the fetal side of the placenta was significantly increased. The study concluded that PM-induced changes in placental perfusion were, at least in part, responsible for the observed reduction in fetal weight.

The present study was designed to establish feasibility for the epidemiologic observations that inhalation exposure during pregnancy to $PM_{2.5}$ leads to PTB and LBW and to determine which (if any) gestational periods are most sensitive for PM-induced LBW, PTB, or both.

Methods

Animals

Seven- to eight-week-old female and male (for breeding purposes only) $B6C3F_1$ mice (Jackson Laboratory) were housed in single-sex pairs upon arrival and were provided food and water

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ad libitum at all times except during concentrated ambient $PM_{2.5}$ (CAPs) exposure. Beginning one day after arrival, estrous cycles were monitored daily for at least two complete normal estrous cycles. On the third proestrus, following two normal cycles, a single female mouse was paired overnight with one male. The next morning, confirmation of successful mating was determined by the presence of a copulatory plug and was considered GD0.5. Mated females were weighed and randomly assigned to one treatment group [i.e., filtered air (FA) control vs. CAPs] and to one of four gestational exposure periods: period 1 (GD0.5–GD5.5); period 2 (GD6.5–GD14.5); period 3 (GD14.5–GD16.5); or period 4 (GD0.5–GD16.5) (Figure 1). A group of pregnant naïve mice ($n=4$) remained in their home cages during the exposure period and served as chamber controls to assure that any observed effects were due solely to treatment rather than to the exposure system itself. When not being exposed, all experimental animals were housed in rooms equipped with HEPA and charcoal filters to remove any ambient particles and gaseous pollutants.

A total of three CAPs exposure experiments were performed between 2012 and 2013: The first (summer of 2012) and second (winter of 2013) exposure examined the effects of maternal CAPs exposure throughout gestation (i.e., period 4) only. The third exposure occurred during the summer of 2013 for the purpose of assessing specific gestational periods of greatest vulnerability to $PM_{2.5}$.

Exposure System

A particle concentrator system was used to collect and concentrate $PM_{2.5}$ for each experiment as described previously (Maciejczyk et al. 2005). Briefly, the system is a modified versatile aerosol concentration enrichment system (VACES) originally developed by Sioutas et al. (1999). The principle of VACES is "condensational growth of ambient particles followed by virtual impaction to concentrate the aerosol" (Maciejczyk et al. 2005; Sioutas et al. 1999). Ambient air was drawn through an Aerotec 2 cyclone inlet that removes the majority of particles >2.5 μm in diameter and was then passed through silica gel and carbon filters to remove excess moisture and organic pollutants. Water-soluble [e.g., sulfur dioxide (SO_2)] and reactive [ozone, nitrogen oxides (NO_x)] gases were removed by the system itself. The PM aerosol was then quickly chilled to ~20°C in a condenser tube. The remaining concentrated particles were then passed over a warmed water bath to restore relative humidity similar to that of ambient air. From there, the CAPs aerosol was divided into three streams: 27% of the particle flow was directed toward Teflon™ filters housed in Harvard impactors (Air Diagnostics and Engineering Inc.) and was used for gravimetric and chemical analysis (described below); 10% of the flow was directed toward a DataRAM™ nephelometer (Thermo Electron Corporation) to allow for continuous monitoring of CAPs mass concentration; the remaining particles were streamed toward the animal exposure chamber. The same system was used for the control mice, which were exposed to house air that passed through HEPA filters, which removed ~98% of ambient particles before entering the VACES inlet.

For each of the exposures, the target CAPs concentration was 150 $\mu g/m^3$; this level is ~10–15 times that of the ambient $PM_{2.5}$ concentration usually found at the New York University (NYU) Sterling Forest (Tuxedo, NY) facility where the ambient $PM_{2.5}$ was collected. The selected concentration was chosen such that a 6-h exposure period, when averaged over a 24-h period, was relevant to that measured in some U.S. urban centers (Samet et al. 2000). Because no energy generation plants or other types of industrial operations are located within 20 miles of the exposure system, CAPs produced by the system was representative of the regional $PM_{2.5}$ background for the megalopolis extending from Virginia to Maine on the eastern coast of the United States. Use of the VACES system neither chemically nor physically modifies the ambient particles collected by the CAPs exposure system (Chen et al. 2005).

Mouse Exposure

Individual mice were placed into single compartments of a 32-compartment stainless steel exposure chamber. The exposure box was covered by a Plexiglass lid through which perforated aluminum tubes delivered CAPs evenly throughout the exposure box (Maciejczyk et al. 2005). Mice were weighed each morning

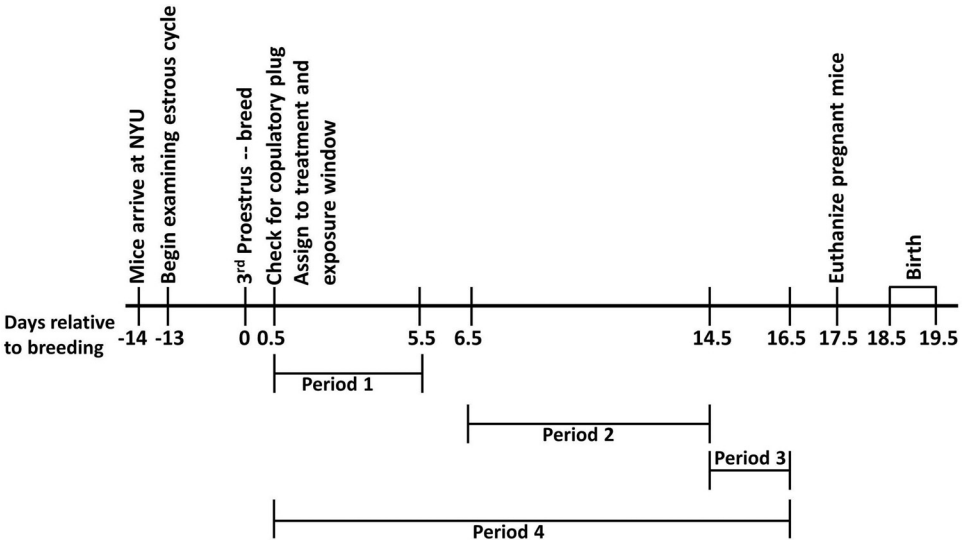

Figure 1. Timeline for inhalation CAPs exposure. Upon arrival, female mice were staged for phase of the estrous cycle. On the third proestrus following two normal cycles, the female was paired with a single male to breed overnight. Upon confirmation of breeding, the female was weighed and assigned to a treatment and to an exposure period. Exposures were 6 h/d, 7 d/wk. Dams were weighed daily before being placed into the exposure box if being exposed or returned to the home cage if not being exposed. Mice from all periods were either euthanized on GD17.5 or allowed to give birth as described in "Methods." Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); NYU, New York University.

before being placed into the exposure box; those that were not being exposed were returned to their cages after weighing. For experiment 3, mice were exposed during one of four exposure periods. To reduce possible effects from differences in PM content between exposures, mice from each period were overlapping (i.e., not in a sequential manner). A subset of pregnant mice ($n = 6–8$) from each gestational exposure period was euthanized on GD17.5 using sodium pentobarbital (150 mg/kg, IP), and the uteri were collected and opened to collect each fetal-placental unit. After all fetal–placental units were excised, the amniotic sacs were carefully opened, the umbilical cords were severed at the fetal-umbilical attachment site, and the umbilical cords and amniotic membranes were dissected away from the placenta. The position of the fetal-placental unit within the uterus was not recorded for these studies. All fetuses and placentas recovered from each dam were weighed, and fetal crown-to-rump length (CRL) was determined using digital calipers. The remaining timed-pregnant dams ($n = 8–17$) in each exposure period were permitted to give birth, and the day of parturition was recorded; each neonate was weighed, and CRL was measured at birth and daily for 21 d, at which point they were weaned.

Starting on GD18.5, cages were checked for the presence of pups starting at 0800 hours. If present, pups were immediately counted and weighed. Alternatively, if no pups were present at that time, mice were checked every 2–3 hours until midafternoon. If pups were not observed on any given day by 1630 hours, dams were checked again the following morning. To avoid data biasing, neonatal weights were collected only after milk was viewed by eye in their stomachs; pups that had not been fed weighed less than those that had been nursed. In circumstances where litters contained more than 10 pups at birth, the number of neonates was culled to 10 on postnatal day (PND) 0. On PND10 and PND21, neonatal anogenital distance (AGD) was measured in both male and female offspring. All procedures using animals were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Genetic Sexing of Fetal Mice

Sexing of GD17.5 fetal mice followed the same protocol as previously described (Blum Het al. 2012). Briefly, a 1-mm section of the fetal tail was clipped from each fetus after weighing, placed into a microcentrifuge tube containing 100 μL digestion buffer [25 mM sodium hydroxide (NaOH)/0.2 mM ethylenediaminetetraacetic acid (EDTA), pH 12.0], and incubated at 95°C for 1 h. Once digested, 100 μL of neutralization buffer (40 mM Tris, pH 5.5) was added to each tube and thoroughly mixed by vortexing. Undigested material was separated via centrifugation ($1,000 \times g$ for 10 min), and the supernatant was collected and diluted 1:100 in ultrapure water. The diluted DNA sample was used as a template for duplex polymerase chain reaction (PCR) using primers for interleukin-3 and the sex-determining region of chromosome Y (SRY) gene. PCR products were separated using 1% agarose gel electrophoresis in tris-acetate-EDTA buffer and were visualized using ethidium bromide staining and ultraviolet light illumination.

Elemental Characterization and Mass Concentration of Collected $PM_{2.5}$ Particles

Using preweighed Teflon filters (37 mm, 0.2 μm pore size; Pall), the mass concentration of CAPs was determined daily; the particle concentration from filtered air (FA) was determined on a weekly basis. Particle-laden filters were equilibrated overnight in a temperature/humidity-controlled weigh room ($21°C \pm 0.5°C$ and $40 \pm 5\%$ relative humidity) and were weighed gravimetrically on an MT5 microbalance (Mettler Toledo). Filters from every third exposure day, as well as lot-matched unexposed blank control filters, were analyzed by X-ray fluorescence spectroscopy (XRF) to determine elemental content using an ARL™ Quant'X EDXRF Analyzer (ThermoScientific).

Statistical Analyses

In all cases, the dam was the experimental unit (Table 1 details sample sizes across experiments and exposure periods). Gestational days of birth, birth weights, fetal body weight, CRL, placental weight, weight-to-length ratio, and anogenital distance were compared using analysis of variance (ANOVA). For the first two experiments, the main effect was treatment. Because no statistical differences were observed across exposure periods for FA-exposed dams in experiment 3, data from all FA-treated dams in this experiment were pooled for statistical analyses and graphical presentations. For data generated from experiment 3, the main effects tested were treatment and exposure period, along with the interaction effect of treatment × exposure period. For measurements of body weight gain (percent change from birth and percent change day-over-day), data from days postpartum were compared between the four CAPs exposure periods and the pooled FA control. When statistically significant differences were observed (ANOVA p-value <0.05), post hoc testing was performed using Fisher's Least Significant Difference (LSD) to identify differences between treatments in the case of experiment 1, or between individual CAPs exposure periods, or in comparison to the pooled FA control in experiment 3. Comparisons of offspring sex ratios between exposure periods, between treatments, or between exposure periods and treatments were performed using χ^2 analysis. All statistical comparisons were performed using SAS (v.9.1.3; SAS Insitute Inc.). Data presented are the means ± standard error (SE) unless otherwise stated.

Results

Physicochemical Analyses of CAPs

Concentrations of CAPs varied moderately between each of the three experiments (Table 2). For the first and second exposures, pregnant mice were exposed to CAPs throughout gestation (GD0.5–GD16.5). The mean CAPs concentration for the first experiment was 15.2 times greater than ambient air levels and 44.3 times higher than FA levels. The CAPs mass concentration for the second experiment was 24.6 times higher than ambient air and 29.1 times higher than FA. For the third experiment, pregnant mice were exposed to CAPs only during one of four preselected gestational exposure periods in an overlapping manner. Compared with the ambient

Table 1. Experimental sample sizes for each treatment in each experiment.

Experiment	Treatment	Total sample size	Number of dams used for GD17.5	Number of dams used for PTB/LBW
Experiment 1	Naïve	4	0	4
	FA	10	0	10
	CAPs	15	0	15
Experiment 2	FA	22	0	22
	CAPs	22	0	22
Experiment 3	Period 1	10	4	6
FA	Period 2	10	4	6
	Period 3	13	4	9
	Period 4	10	5	5
CAPs	Period 1	13	5	8
	Period 2	13	5	8
	Period 3	13	5	8
	Period 4	16	5	11

Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); FA, fitered air; GD, gestation day; LBW, low birth weight; PTB, preterm birth.

Table 2. Average daily CAPs concentrations.

	Experiment 1[a]	Experiment 2[b]	Experiment 3[c]			
	Period 4	Period 4	Period 1	Period 2	Period 3	Period 4
Treatment	(GD 0.5 – 16.5)	(GD 0.5 – 16.5)	(GD 0.5 – 5.5)	(GD 6.5 – 14.5)	(GD 14.5 – 16.5)	(GD 0.5 – 16.5)
FA	3.7 ± 1.7[d]	3.9 ± 2.6		2.7 ± 1.6		
CAPs	163.8 ± 100.0	113.4 ± 93.7	177.5 ± 104.7	192.5 ± 96.2	171.3 ± 94.1	173.4 ± 92.2
Ambient[e]	10.9 ± 6.5	4.7 ± 3.4		11.6 ± 6.1		

Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); FA, fitered air; GD, gestation day.
[a]Experiment 1 occurred during summer 2012.
[b]Experiment 2 occurred during winter 2013.
[c]Experiment 3 occurred during summer 2013.
[d]Values are mean daily concentrations ($\mu g/m^3$) $\pm$ standard deviation (SD) for each particular gestational time frame.
[e]Ambient concentrations provided for comparison only.

$PM_{2.5}$ concentrations measured during these same periods, the mean CAPs concentrations were 15.3-, 16.6-, 14.8-, and 14.9 times higher for periods 1–4, respectively; compared with FA, CAPs concentrations were 65.7-, 71.3-, 63.4-, and 64.2 times higher for the same gestational periods.

Elemental analyses were performed on particle-laden filters collected every third exposure day from all three experiments; the results are shown in Table 3. Elemental levels, with the exceptions of copper (Cu), zinc (Zn), bromine (Br), and lead (Pb), were greater during the summer exposures than during the winter months. For both summer experiments (experiments 1 and 3), the 10 most abundant CAPs-associated elements were sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), sulfur (S), potassium (K), calcium (Ca), titanium (Ti), iron (Fe), and bromine (Br). For the winter exposure (experiment 2), the most abundant CAPs-associated elements were the same as those measured during the summer except that Al and Si were replaced by Cu and Zn. Elements collected on filters collected from the FA exposure line did not show significant variability across the three experiments.

Exposure of Pregnant Mice to CAPs Results in PTB and LBW

In the first experiment (summer 2012), pregnant mice exposed to CAPs (163.8 $\mu g\,CAPs/m^3$) throughout gestation (GD0.5–GD16.5) demonstrated a 0.5-d reduction ($p = 0.0018$) in gestational duration compared with both naïve and FA-exposed mice (Figure 2A). A significant ($p = 0.0059$) decrease (11.4%) in birth weight was also observed for offspring born prematurely (Figure 2B). There were no significant differences in gestational duration or birth weight between naïve and FA-exposed groups, demonstrating that exposure to CAPs, specifically, rather than confinement stress, was responsible for the observed effects. The results from the second experiment (winter 2012), also encompassing CAPs exposure throughout gestation, supported the PTB and LBW findings from the first exposure despite the difference in season. In this case, pregnant mice exposed to CAPs at a lower concentration than in the first experiment (113.4 $\mu g/m^3$ vs. 163.8 $\mu g/m^3$, respectively) had a reduction of ~ 0.3 d ($p = 0.0423$) in pregnancy duration compared with FA-exposed mice (Figure 2C) that was also associated with an 8.8% decrease ($p = 0.0005$) in average litter birth weight (Figure 2D).

Assessments of litter sizes and sex ratios were performed for all experiments (Table 4). Across all experiments and treatments, the average litter size was 8.3 pups per litter, with an overall range of 3–12. Within each experiment, there were no significant differences ($p > 0.05$) between treatments for litter size, numbers of a given sex (determined by ANOVA) or sex ratios (determined by χ^2 analysis). In experiment 3, no statistically significant differences were observed across exposure periods within treatment groups or between treatment groups for each period.

Effects of Exposure of Pregnant Mice to CAPs on Fetal Weight, Fetal CRL, and Placental Weight (Experiment 3)

Based on the observations from the first two experiments, follow-up studies were performed in the third experiment that focused on fetal, neonatal, and placental parameters. Fetal body weights examined on GD17.5 in experiment 3 revealed that effects were dependent ($p = 0.0115$) upon the period during pregnancy when exposure to CAPs occurred (Figure 3A). Fetuses collected at GD17.5 from dams exposed to CAPs during only the fetal growth period (period 3) and throughout gestation (period 4) at similar CAPs concentrations (171.3 vs. 173.4 $\mu g/m^3$, respectively) were 8.1% and 7.7% lighter, respectively, than GD-matched counterparts from FA-exposed dams. Moreover, maternal CAPs exposure during periods 1 (177.5 $\mu g/m^3$), 3, and 4 led to significant ($p = 0.0468$) decreases in CRL of 2.7%, 5.0%, and 1.8%, respectively (Figure 3B). In addition, maternal exposure to CAPs resulted in significant ($p = 0.0138$) changes in placental weight. Maternal exposure to CAPs during the fetal growth period alone (period 3) resulted in an 8.1% decrease in placental weight. When exposure to CAPs occurred throughout pregnancy (period 4), a 3.8% increase in placental weight was observed (Figure 3C). Exposures that occurred only during placentation/organogenesis (period 2) had no effect on fetal weight, CRL, or placental weight.

Effects of Exposure of Pregnant Mice to CAPs on Newborn Body Weight and CRL

In the third experiment carried out in the summer of 2014, pregnant mice were exposed to CAPs during one of four gestational periods. CAPs exposure caused an exposure period–dependent decrease ($p = 0.0003$) in gestational duration. As shown in Figure 4A, no change in gestational duration was observed for mice born to mothers exposed only before implantation (period 1). In contrast, offspring from dams exposed to CAPs during either organogenesis (period 2) or growth (period 3) or throughout gestation (period 4) demonstrated reduced gestational duration of 0.4, 0.5, or 0.4 d, respectively. Birth weights were significantly ($p = 0.0003$) reduced by 10.3%, 9.8%, and 10.3% (compared with controls) following maternal exposure to CAPs during periods 1, 2, and 4, respectively, whereas exposure to CAPs only during the fetal growth period had no effect on birth weight (Figure 4B).

At birth, CRL was significantly ($p < 0.0001$) decreased irrespective of the maternal exposure period in experiment 3 (Figure 4C). CRLs were reduced by 4.0%, 3.9%, 3.3%, and 4.6% for CAPs exposure periods 1–4, respectively. Decreased size-for-gestational age (SGA; weight/length) was observed in offspring

Table 3. Concentrations of various elements found on collection filters identified by XRF.

Element	FA: Summer 2012	FA: Winter 2013	FA: Summer 2013	Ambient[b]: Summer 2012	Ambient: Winter 2013	Ambient: Summer 2013	CAPs: Summer 2012	CAPs: Winter 2013	CAPs: Summer 2013
Sodium (Na)	16.8 ± 10.0[a]	−12.3 ± 25.2	56.9 ± 32.6	188.3 ± 161.6	110.0 ± 60.6	162.6 ± 88.0	1411.6 ± 1871.8	1154.5 ± 601.1	1593.6 ± 1003.2
Magnesium (Mg)	0.7 ± 5.1	−1.0 ± 5.4	15.8 ± 8.6	45.4 ± 30.3	20.0 ± 11.1	46.7 ± 25.8	349.6 ± 305.1	253.5 ± 253.5	510.8 ± 353.8
Aluminum (Al)	−9.4 ± 11.1	40.7 ± 75.9	4.7 ± 11.6	10.3 ± 22.5	−5.2 ± 12.1	46.5 ± 96.9	241.2 ± 205.1	−34.0 ± 70.0	755.5 ± 1589.6
Silicon (Si)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 3.2	0.0 ± 0.0	50.1 ± 186.4	329.2 ± 573.5	0.0 ± 0.0	1376.5 ± 3426.5
Sulfur (S)	10.2 ± 4.9	13.1 ± 0.8	10.9 ± 4.5	686.0 ± 755.6	402.3 ± 109.2	1101.1 ± 803.8	10057.4 ± 12697.3	6750.3 ± 2262.1	18000.9 ± 13109.9
Potassium (K)	4.3 ± 1.5	1.4 ± 0.6	1.5 ± 2.6	31.6 ± 11.4	33.4 ± 8.5	52.0 ± 33.9	398.2 ± 204.1	493.0 ± 74.6	708.7 ± 474.2
Calcium (Ca)	8.7 ± 6.0	5.1 ± 2.6	4.0 ± 1.7	34.7 ± 26.5	22.5 ± 10.1	40.9 ± 32.5	445.7 ± 335.9	294.9 ± 139.6	538.9 ± 516.2
Titanium (Ti)	3.2 ± 1.8	1.6 ± 1.1	2.7 ± 1.2	14.1 ± 5.3	14.6 ± 3.8	17.7 ± 12.5	347.7 ± 132.8	472.5 ± 177.5	1000.5 ± 1110.0
Vanadium (V)	0.1 ± 0.4	0.3 ± 0.0	0.1 ± 0.3	1.3 ± 1.4	0.3 ± 1.0	0.7 ± 1.0	4.1 ± 11.3	3.9 ± 8.7	7.2 ± 7.8
Chromium (Cr)	0.7 ± 0.4	−0.2 ± 0.4	0.0 ± 0.3	−0.3 ± 2.3	−0.4 ± 1.2	−0.2 ± 0.9	2.4 ± 7.3	−1.8 ± 4.2	4.5 ± 8.3
Manganese (Mn)	0.0 ± 0.2	0.2 ± 0.1	−0.1 ± 0.3	1.3 ± 1.2	0.5 ± 1.0	1.3 ± 1.2	14.8 ± 8.8	11.9 ± 8.5	23.2 ± 18.5
Iron (Fe)	5.4 ± 5.3	2.9 ± 1.4	0.3 ± 0.5	35.4 ± 18.6	19.9 ± 4.4	58.3 ± 62.4	473.0 ± 248.2	292.1 ± 62.3	905.5 ± 1067.9
Nickel (Ni)	0.2 ± 0.1	0.2 ± 0.4	0.2 ± 0.3	1.0 ± 0.6	0.3 ± 0.7	1.6 ± 3.4	8.7 ± 5.0	7.7 ± 3.4	7.7 ± 6.1
Copper (Cu)	0.5 ± 0.3	0.7 ± 0.3	0.4 ± 0.5	1.8 ± 0.9	0.8 ± 0.5	1.5 ± 1.5	11.6 ± 7.3	27.1 ± 22.8	21.0 ± 9.6
Zinc (Zn)	1.4 ± 0.4	0.7 ± 0.8	1.7 ± 2.5	4.1 ± 1.4	4.7 ± 3.3	9.4 ± 17.3	48.7 ± 31.1	80.2 ± 31.9	70.9 ± 39.7
Bromine (Br)	0.1 ± 0.6	0.4 ± 1.5	0.4 ± 0.9	6.1 ± 8.2	8.3 ± 8.2	6.3 ± 6.1	108.9 ± 119.6	214.5 ± 109.6	156.1 ± 84.2
Strontium (Sr)	0.1 ± 0.4	0.2 ± 0.4	0.3 ± 0.5	0.3 ± 1.3	0.6 ± 0.8	0.8 ± 1.2	6.8 ± 5.2	7.7 ± 7.8	13.9 ± 11.8
Barium (Ba)	−0.3 ± 0.9	2.1 ± 5.0	1.4 ± 2.0	3.0 ± 5.5	−1.0 ± 1.6	1.9 ± 5.1	7.5 ± 28.0	5.1 ± 18.3	25.9 ± 44.2
Erbium (Er)	0.8 ± 0.5	1.0 ± 0.5	0.3 ± 0.6	1.1 ± 1.6	0.4 ± 1.0	0.8 ± 1.7	5.3 ± 6.6	8.3 ± 7.4	13.8 ± 8.2
Lutetium (Lu)	0.6 ± 0.9	0.6 ± 0.9	0.6 ± 0.6	1.4 ± 1.4	2.6 ± 1.2	2.7 ± 3.8	18.6 ± 9.3	24.4 ± 6.9	24.9 ± 11.0
Lead (Pb)	−0.2 ± 0.1	0.2 ± 0.5	0.0 ± 0.2	−0.2 ± 0.8	0.4 ± 0.9	1.6 ± 1.3	12.7 ± 14.7	17.1 ± 9.7	15.4 ± 10.8

Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); FA, filtered air; GD, gestation day; XRF, X-ray fluorescence spectroscopy.
[a]Data presented are mean ng/m^3 ± standard deviation (SD) from filters collected during each exposure. Each mean represents 4–18 filters across each respective exposure period that were analyzed via XRF as described in "Methods."
[b]Data presented for ambient are for informational purposes only. No mice were exposed to these particles.

born to dams exposed only during periods 1, 2, and 4; differences in SGA were reflected by decreases of 7.4%, 6.1%, and 6.0%, respectively, compared with FA-exposed mice ($p = 0.0054$). Exposure of dams to CAPs only during period 3 had no effect on neonatal size for gestational age (Figure 4D).

Maternal CAPs Exposure Did Not Alter Postnatal Weight Gain in Neonatal Offspring (Experiment 3)

The effects of prenatal CAPs exposure on neonatal weight gain were dependent upon the method used to calculate the outcome. When weight gain was calculated as increase in body weight over time relative to birth weight, no differences ($p > 0.05$) in growth rates were observed compared with the FA control (Figure 5A). Furthermore, calculation of day-to-day percentage body weight gain also revealed no clear CAPs exposure–related effects ($p > 0.05$) (Figure 5B).

Effects of CAPs Exposure on Body Length and AGD on PND10 and PND21 (Experiment 3)

On PND10, male offspring born to dams that were exposed to CAPs only prior to implantation (period 1) displayed a 2.9% decrease ($p = 0.0138$) in CRL compared with time-matched FA-exposed dams (Figure 6A). In contrast, CRL was significantly ($p = 0.0263$) increased by 1.8% at PND21 in males born to dams exposed to CAPs during mid- to late pregnancy (i.e., period 3) (Figure 6B).

Compared with the FA-exposed controls, offspring from mothers exposed to CAPs during early and mid-pregnancy (i.e., periods 1 and 2) had significantly ($p = 0.0088$) shorter AGDs (10.8%) at PND10. The reduction in AGD for male offspring born to dams exposed to CAPs throughout pregnancy (period 4) was slightly less dramatic (8.8%), although the reduction remained significant (Figure 6C). The CAPs-induced reduction in AGD observed in male offspring at PND10 persisted ($p = 0.0063$) until PND21, but only for those offspring whose mothers were exposed either before implantation or throughout gestation (periods 1 and 4), in which case AGDs were reduced by 5.4% and 4.3%, respectively (Figure 6D).

Similar to that observed for male offspring, maternal CAPs exposure caused significant ($p = 0.0403$) decreases in female CRL. Exposure during period 1 decreased CRL in female offspring on PND10 by 2.4% (Figure 7A). However, by weaning on PND21, female CRL was indistinguishable ($p = 0.2519$) from that of their sex-matched control offspring (Figure 7B). The differences in anogenital distance in female offspring in response to maternal CAPs exposure were more dramatic than those observed in their age-matched male counterparts because AGD was significantly ($p = 0.0001$) reduced by exposure during all exposure periods (Figure 7C). However, AGDs reached control values by PND21, but only in female offspring exposed to CAPs during mid- and late pregnancy (periods 2 and 3); CAPs-induced changes in AGD in offspring exposed during early pregnancy (period 1) and throughout gestation (period 4) persisted ($p = 0.0490$) over time (Figure 7D).

Discussion

There were two main goals of these studies: *a*) Provide experimental evidence to support the human epidemiologic literature linking both PTB and LBW to inhalation exposure of $PM_{2.5}$ during pregnancy at concentrations relevant to many urban centers; *b*) determine whether CAPs-induced PTB, LBW, or both were linked to exposure during a specific gestational period. The 24-h National Ambient Air Quality Standard (NAAQS) for $PM_{2.5}$ concentration established in 2012 by the U.S.

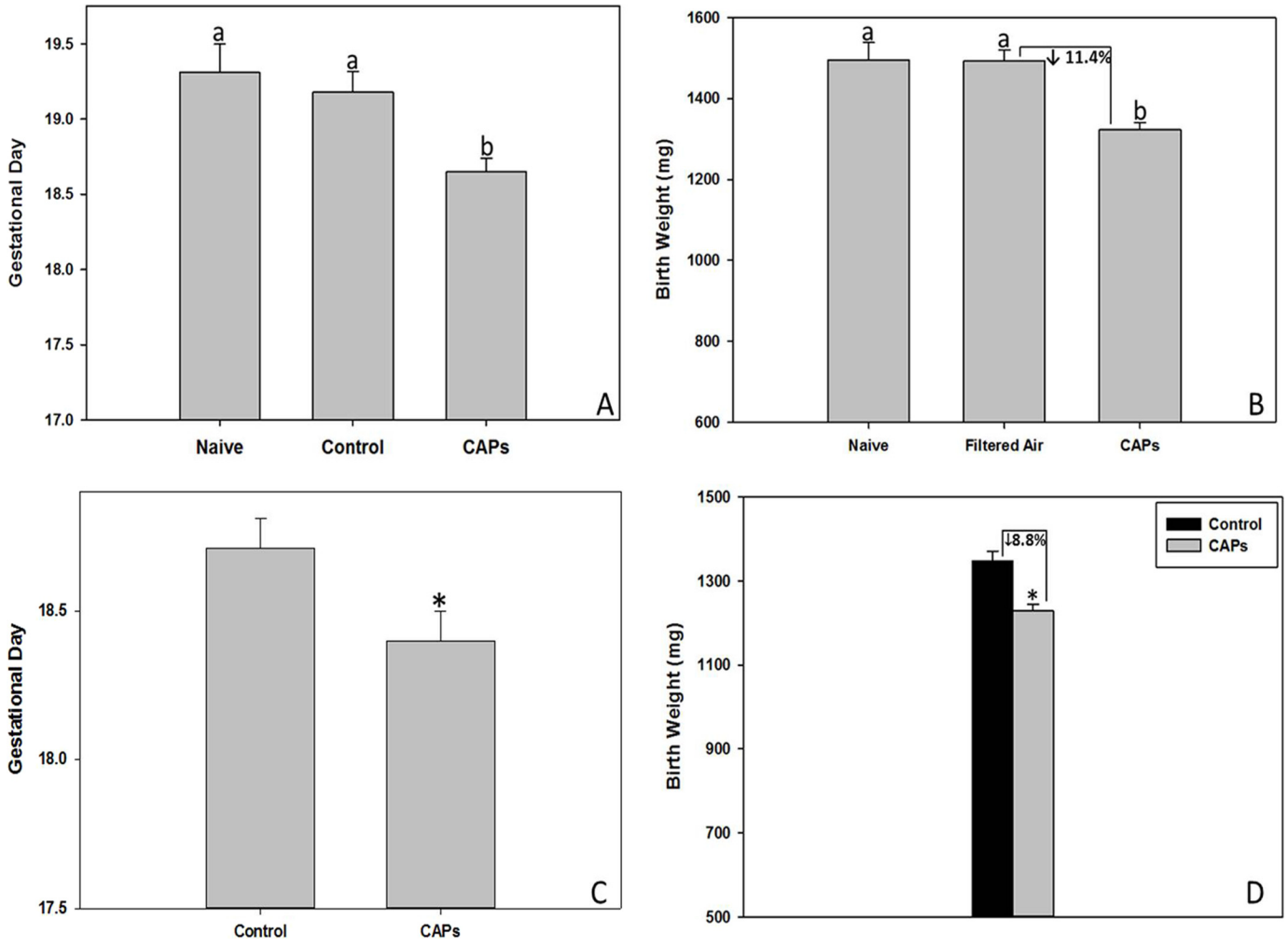

Figure 2. Maternal exposure to inhaled CAPs results in preterm birth and low birth weight. Dams were exposed to CAPs during period 4 (GD0.5–16.5) and were allowed to give birth. Data are from experiment 1 (*A*, *B*) and experiment 2 (*C*, *D*). In experiment 1, some naïve dams ($n=4$) were used to control for changes resulting from the exposure system. Data for experiment 1 are the means ± standard error (SE) from $n=10$ (FA) or $n=15$ (CAPs); for experiment 2, $n=22$ for each treatment. In all panels, the treatment effect is significant [analysis of variance (ANOVA) $p<0.05$]. Bars in panels *A* and *B* with different letters are significantly different based on Fisher's Least Significant Difference (LSD) post hoc testing. Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); FA, filtered air. *$p<0.05$ based on ANOVA.

Environmental Protection Agency (EPA) is 35 μg $PM_{2.5}/m^3$ (U.S. EPA 2012, 2013). Although the time-weighted average (TWA) CAPs concentrations used in some of these experiments exceeded the U.S. EPA standard (the concentration in experiment 1 was 41 μg/m^3 and that in experiment 3 ranged from 42.8–48.2 μg/m^3 over the designated periods), the CAPs levels are nevertheless relevant to many U.S. and global cities that often exceed the NAAQS. In 2006, >200 U.S. counties were surveyed,

Table 4. Average litter size and sex breakdown by experiment, treatment, and exposure period.

Experiment/treatment	Treatment/gestational period	Litter size	Mean number of males	Mean number of females	% Male	% Female
Experiment 1	Naïve	8.2 ± 1.5[a]	4.3 ± 1.7	4.0 ± 0.8	50.0 ± 14.3	50.0 ± 14.3
	FA	7.8 ± 1.9	3.4 ± 0.7	4.3 ± 2.0	45.8 ± 14.3	54.2 ± 20.7
	CAPs	7.9 ± 2.1	3.9 ± 1.2	3.8 ± 2.0	51.8 ± 16.1	48.2 ± 17.3
Experiment 2	FA	8.7 ± 1.3	4.2 ± 1.3	4.5 ± 1.6	49.0 ± 15.4	51.0 ± 15.4
	CAPs	8.3 ± 1.3	3.8 ± 1.7	4.5 ± 1.7	45.9 ± 16.4	54.1 ± 16.4
Experiment 3	Period 1	9.0 ± 0.6	4.2 ± 1.5	4.8 ± 1.3	46.1 ± 15.0	53.9 ± 15.0
FA	Period 2	8.5 ± 1.6	5.0 ± 1.6	3.2 ± 1.8	62.7 ± 18.5	37.3 ± 18.5
	Period 3	9.0 ± 1.3	5.0 ± 1.4	4.5 ± 1.4	52.5 ± 12.9	47.5 ± 12.9
	Period 4	8.4 ± 0.7	4.4 ± 1.7	4.0 ± 2.0	53.0 ± 22.0	47.0 ± 22.0
	Across periods	8.7 ± 1.1	4.6 ± 1.5	4.2 ± 1.7	53.2 ± 16.9	46.8 ± 16.9
CAPs	Period 1	8.7 ± 1.0	4.0 ± 1.8	5.0 ± 1.9	44.6 ± 21.5	55.4 ± 21.5
	Period 2	8.3 ± 1.0	4.9 ± 1.7	3.4 ± 1.8	59.5 ± 20.8	40.5 ± 20.8
	Period 3	7.2 ± 2.7	4.6 ± 0.9	2.6 ± 1.5	65.6 ± 16.6	34.4 ± 16.6
	Period 4	8.3 ± 1.3	4.0 ± 1.2	4.0 ± 1.7	50.9 ± 17.7	49.7 ± 17.7
	Across periods	8.1 ± 1.7	4.4 ± 1.4	3.8 ± 1.8	57.6 ± 19.7	45.4 ± 19.7

Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); FA, fitered air.
[a]Data presented are means ± standard deviation (SD) from all litters generated in these experiments. Fetal mice were sexed using polymerase chain reaction (PCR) as described in "Methods," and neonatal mice were sexed by visual observation on postnatal day 8. Exposure treatment groups were assessed within experiment, and no significant differences were observed.

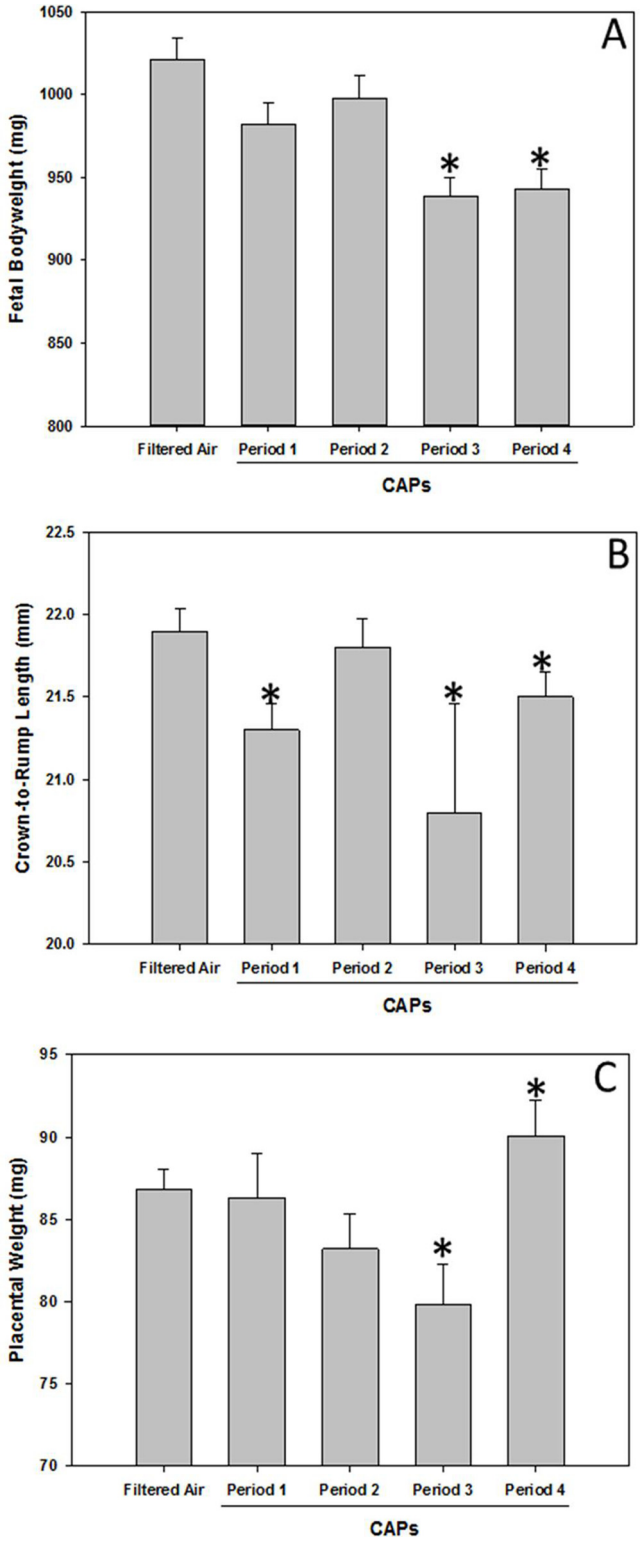

Figure 3. Exposure of pregnant mice to CAPs during different exposure periods (experiment 3) is associated with decreased body weight (*A*), decreased CRL (*B*), and altered placental weight (*C*) on GD17.5. The results from analysis of variance (ANOVA) showed significant differences ($p < 0.05$) among the groups for each endpoint which was followed by Fisher's Least Significant Difference (LSD) post hoc testing to determine differences compared with FA. Data are the means ± standard error (SE) from $n = 5$ dams from each CAPs exposure period or $n = 16$ from the pooled FA control dams. Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); CRL, crown-to-rump length; FA, filtered air; GD, gestational day. *$p < 0.05$ compared with FA dams based on post hoc testing.

and of these, 53 had 24-h $PM_{2.5}$ levels that exceeded the standard (Yip et al. 2011). Many cities throughout the world also have documented levels of $PM_{2.5}$ far exceeding the U.S EPA standard. For example, the daily average $PM_{2.5}$ concentration for Beijing, China in 2013 was 90 $\mu g\, PM_{2.5}/m^3$ (Huang et al. 2014), and >10 other Chinese cities registered even higher concentrations.

In addition to respiratory and cardiovascular health concerns associated with exposure to elevated $PM_{2.5}$ levels, epidemiologic data demonstrate an association between exposure to ambient $PM_{2.5}$ and obstetric consequences including PTB and LBW (Lewandowski et al. 2013; Li et al. 2014). Given the numbers of women of reproductive age worldwide who are exposed daily to elevated $PM_{2.5}$ levels, studies such as these are critical for informing health policy and for better understanding the mechanisms behind these comorbidities.

The gestational time frames selected for $PM_{2.5}$ exposures in these studies were based on recommendations by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) testing guidelines (http://www.ich.org) for predicting reproductive/developmental toxicity in animals. These same time points are highly translatable to humans and represent times during human pregnancy when the developing offspring is most vulnerable to toxic insult (Figure 8). Each specific gestational period of mouse development/growth selected for study represents a critical time period during pregnancy, including *a*) fertilization and implantation (period 1); *b*) placental development/vascularization/nutrient transport and embryonic organogenesis (period 2); and (*c*) placental maturation and rapid fetal growth (period 3). A fourth gestational exposure period that covered all three of the abovementioned periods was also included in experiment 3. In the studies here, period 1 (i.e., GD0.5–GD5.5) corresponds to GD0–GD7–12 in humans, which is the time period during which preimplantation events occur. Period 2 in the mouse (GD6.5–GD14.5) encompasses postimplantation events, including formation and maturation of the placenta and the completion of organogenesis, that occur in humans through the 12th–14th week of gestation, defining the first trimester. The second and third trimesters of human pregnancy align with period 3 (GD14.5 to parturition range) in mice as rapid fetal growth occurs, and the lungs become fully functional.

Normal gestation in humans is 38–40 wk, and birth is considered preterm if it occurs before 37 wk. For the particular mouse strain used here, normal term is approximately 19–19.5 days. Shortening the mouse gestational term by 0.5 d, as seen following maternal exposure to $PM_{2.5}$ during the entire gestational period, corresponds to an approximately 1-wk decrease in humans, thus placing them into the preterm category.

The magnitude of decreases in pregnancy duration and birth weight observed in the summer exposures (experiments 1 and 3) compared with that observed in the winter exposure (experiment 2) suggests that particle concentration and relative compositions are important. In this study, the metal composition (both absolute and relative) and the particle mass differed depending on the season in which the mice were exposed. Schwab et al. (2004) reported that $PM_{2.5}$ concentrations from various regions in the state of New York vary throughout the year. Early studies by Thurston et al. (1994) also reported that metal components of $PM_{2.5}$ can show seasonal fluctuations between winter and summer.

Many of the $PM_{2.5}$-associated metals identified in the present study have been implicated as risk factors for LBW in the northeastern and mid-Atlantic regions of the United States. Positive associations have been shown with each interquartile increase of Al, Ca, Ni, Ti, and Zn, with risk ratios ranging from 3.0 for Ca {46 ng/m^3 [95% confidence interval (CI): 1.36–4.3]} to 5.7 for

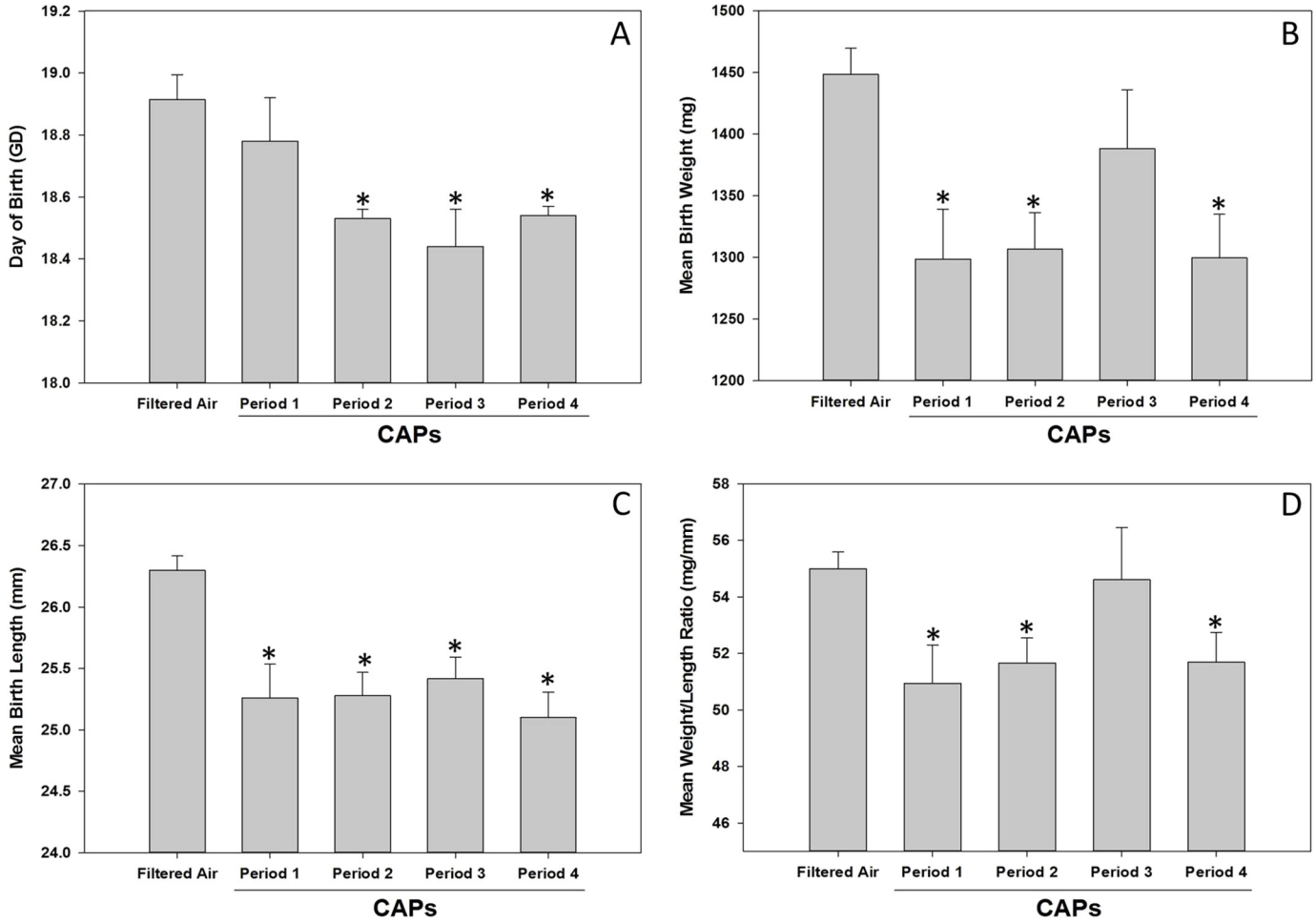

Figure 4. Maternal exposure to inhaled CAPs during different periods of pregnancy in experiment 3 as described in "Methods" are associated with PTB (*A*), LBW (*B*), decreased CRL (*C*) and decreased SGA (*D*). The results from analysis of variance (ANOVA) showed significant differences ($p < 0.05$) among the groups for each end point; ANOVA was followed by Fisher's Least Significant Difference (LSD) post hoc testing to determine differences compared with FA. Data are the means ± standard error (SE) from $n = 8$–11 dams for CAPs-exposed mice during periods 1 – 4. Because no differences were observed among the four periods for FA control values, the values were pooled ($n = 26$). Note: CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); CRL, crown-to-rump length; FA, filtered air; LBW, low birth weight; PTB, preterm birth; SGA, size for gestational age. *$p < 0.05$ compared with FA dams based on post hoc testing.

nickel (Ni) [6 $\mu g/m^3$ (95% CI 2.7–8.8)] (Ebisu and Bell 2012). In full-term infants, LBW was associated with maternal exposure to $PM_{2.5}$ with the following average levels of metallic components: vanadium (V) (4.3 ng/m^3), S (0.83 $\mu g/m^3$), Fe (0.16 $\mu g/m^3$), Ti (10 ng/m^3), manganese (Mn) (3.3 ng/m^3), Br (4.4 ng/m^3), Zn (15 ng/m^3), and Cu (9 ng/m^3) (Basu et al. 2014). In our toxicological study, the aforementioned metal concentrations associated with epidemiological studies were exceeded in all experiments (with the exception of V in experiment 2), suggesting that PM-associated metals could be playing a role in the observed toxicity. However, further research is necessary to better understand the role of PM-associated metals in causing LBW in the present scenario.

In this study, PTB was associated not only with CAPs exposure throughout pregnancy but also with exposure during particular gestational periods. When CAPs exposure occurred only prior to blastocyst implantation, no effects were observed on gestational duration compared with the control. This result supports epidemiologic findings suggesting that $PM_{2.5}$-induced PTB is associated with exposure occurring later in pregnancy (i.e., during the second or third trimester) (Ha et al. 2014). However, these findings are in contrast to those of Symanski et al. (2014) who demonstrated that exposure to a 10 $\mu g/m^3$ increase in $PM_{2.5}$ concentration during the first 4 wk of pregnancy, the time of human blastocyst implantation, was associated with a 73% increased risk for PTB. A study by Rapazzo et al. (2014) revealed that risk for PTB was most closely associated with exposure to $PM_{2.5}$ during the fourth week of gestation (i.e., just after implantation, corresponding to the early part of period 2 in the present study); exposure to $PM_{2.5}$ during the week of birth and during the last two weeks before birth in that study also resulted in early delivery. The authors concluded that exposures to $PM_{2.5}$ around the time of implantation or near birth were the highest risk for PTB.

In the present study, maternal exposure to $PM_{2.5}$ during any gestational period other than the fetal growth phase (period 3) resulted in LBW. Harris et al. (2014) correlated $PM_{2.5}$ concentrations with LBW and found that U.S. states with the highest $PM_{2.5}$ concentrations such as New York (average $PM_{2.5}$ concentration of 13 $\mu g/m^3$) also had the highest rates of LBW (2.6%). In contrast, Utah and Minnesota (average $PM_{2.5}$ concentration of 9 $\mu g\, PM_{2.5}/m^3$) had the lowest rates of LBW (2.1% and 1.9%, respectively). The same study also showed that in New York State, LBW was linked to $PM_{2.5}$ exposure levels during each of the three trimesters as well as to full-term exposure. For all states examined, the highest risk for LBW was associated with

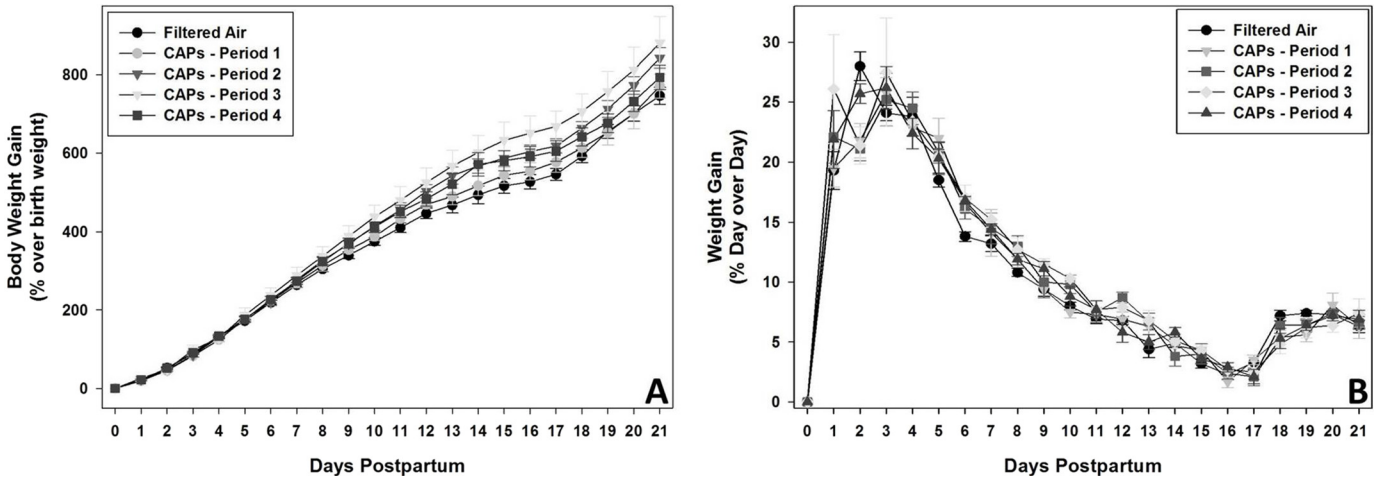

Figure 5. Exposure of pregnant mice does not affect growth rates of offspring (experiment 3). Neonatal body weight gain was computed as a percentage over birth weight (*A*) or daily body weight gain (percent day-to-day gain) (*B*). Analysis of percent weight gain compared to birth weight (*A*) showed no significant differences by ANOVA ($p > 0.05$) for the interaction of treatment and time. Comparison of weight gain day-to-day (*B*) also revealed no significant differences among the groups when data were analyzed by day postpartum. Data are means ± SE from 8–11 dams for each CAPs exposure Period and 26 dams for the pooled FA controls.

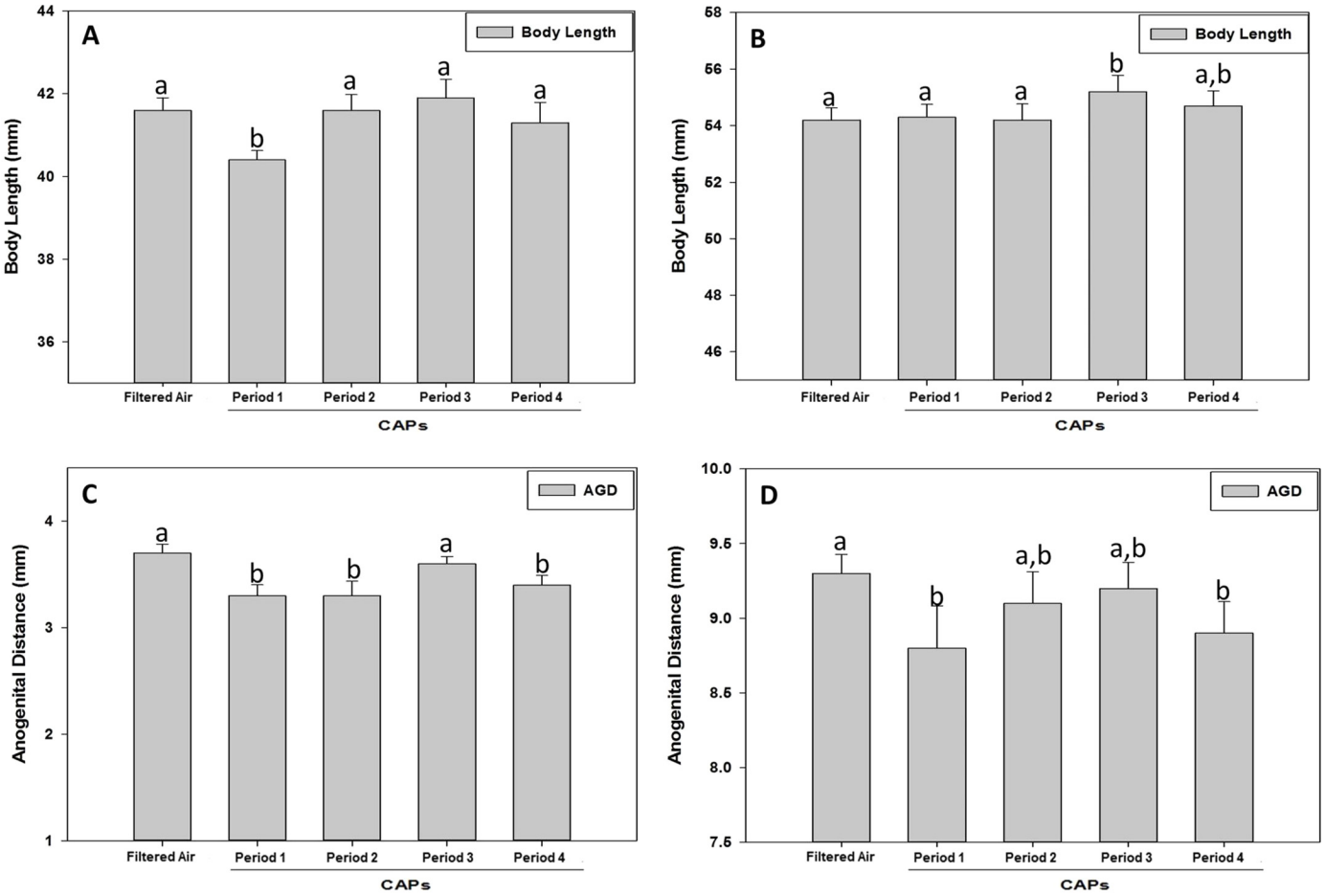

Figure 6. Exposure of pregnant mice to CAPs during different pregnancy periods results in alterations in CRL and AGD in male offspring on PND10 and PND21. CRLs of male offspring were measured on PND10 and PND21 (*A*, *B*), and AGDs were measured at these same time points (*C*, *D*). The results from analysis of variance (ANOVA) showed significant differences ($p < 0.05$) among the groups for each end point; ANOVA was followed by Fisher's Least Significant Difference (LSD) post hoc testing to determine specific differences among the groups. Data presented are the means ± standard error (SE) from $n = 8 - 11$ dams for each CAPs exposure period and $n = 26$ dams for the pooled FA controls. Bars with different letters are significantly different from one another ($p < 0.05$). Note: AGD, anogenital distance; CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); CRL, crown-to-rump length; FA, filtered air; PND, postnatal day.

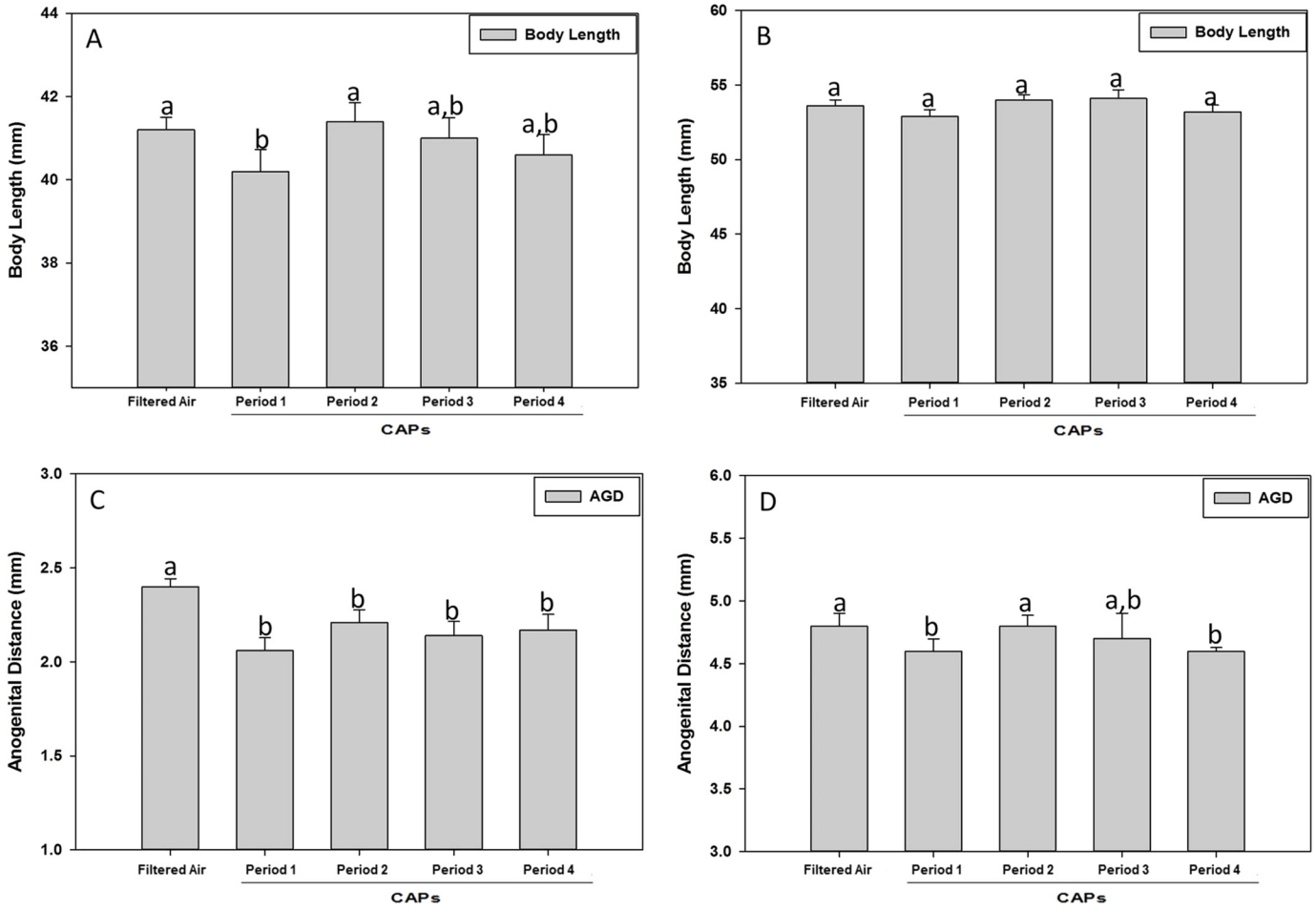

Figure 7. Exposure of pregnant mice to CAPs during different pregnancy periods results in alterations in CRL and AGD in female offspring on PND10 and PND21. CRLs of female offspring were measured on PND10 and PND21 (*A*, *B*), and AGDs were measured at these same time points (*C*, *D*). The results from analysis of variance (ANOVA) showed significant differences ($p < 0.05$) among the groups for each endpoint; ANOVA was followed by Fisher's Least Significant Difference (LSD) post hoc testing to determine specific differences among the groups. Data presented are the means ± standard error (SE) from $n = 8 - 11$ dams for each CAPs exposure period and $n = 26$ dams for the pooled FA controls. Bars with different letters are significantly different from one another ($p < 0.05$). Note: AGD, anogenital distance; CAPs, concentrated ambient $PM_{2.5}$ (fine-sized particulate matter); CRL, crown-to-rump length; FA, filtered air; PND, postnatal day.

exposure during the first trimester [odds ratio (OR) = 1.018 (95% CI 1.013, 1.022)] and full-term exposure [OR = 1.030 (95% CI 1.022, 1.037)], with exposure during the second and third trimesters resulting in a lower risk. Our experimental animal data support the human epidemiologic studies demonstrating that maternal exposure to high $PM_{2.5}$ levels between implantation and the end of the second trimester in humans is the most sensitive time frame for suppressing birth weight.

Following implantation, placentation is the next major milestone during fetal development. In the present study, placental weight was decreased significantly with maternal exposure to $PM_{2.5}$ during the gestational window of rapid fetal growth (i.e., period 3). In contrast, exposure to $PM_{2.5}$ throughout gestation increased placental weight compared with FA controls. To our knowledge, these findings are the first to demonstrate that $PM_{2.5}$-induced changes in placental weight are based upon the timing of exposure in an animal model. In a study by Veras et al. (2008), whole-body exposure of mice to $PM_{2.5}$ (24-h average level of 27.5 $\mu g/m^3$) from traffic in São Paulo, Brazil, before breeding (exposed 24 h/d from 20 d of age to 6 wk of age) or during pregnancy alone decreased fetal weight ($\sim 23\%$) on GD18. In that study, decreased fetal weight was associated with reduced vasculature volumes, luminal diameters, and surface areas of the blood spaces on the maternal face of the placenta. The authors suggested that exposure to traffic-related $PM_{2.5}$, either before conception or immediately after breeding, caused restrictions in maternal blood circulation through the placenta, which led to reduced birth weights. Increased fetal capillary surface area observed in that study was considered by the authors to be a result of the release of fetal "factors" that enhanced blood circulation through the placenta or enlargement of the surface areas available for nutrient exchange, or a combination of the two, to compensate for maternal vasoconstriction that may have resulted from $PM_{2.5}$-induced inflammation (de Melo et al. 2015). Because the mouse placenta continues to grow throughout fetal development, mechanisms similar to those described above may have been responsible for the placental changes observed in our study. It is possible that maternal blood circulation to the placenta experienced greater restriction in mice that began their exposure in period 3 owing to increased amounts of maternal systemic inflammatory mediators.

In contrast to our observations from period 3, placentas from dams exposed to $PM_{2.5}$ throughout pregnancy (i.e., period 4) were heavier than those recovered from their FA control counterparts on GD17.5. As suggested by Veras et al. (2008), increased placental weight could have resulted from

Mouse (GD)	Event	Human (GD)	
0	Fertilization	0	1st Trimester
1-2.5	Cleavage	2-3	
2-4	Blastocyst stage	2-4	
4.5-5	Implantation	6-12	
6-14	Placentogenesis	28-91	
14	Organogenesis complete	84-98	
14-17	Fetal and Placental growth	99-196	2nd Trimester
17-birth	Accelerated Fetal Growth	197-birth	3rd Trimester
19-21	Birth	~294	

Figure 8. Alignment of mouse reproductive timeline to that of humans from the beginning of pregnancy through parturition. This table is based on Theiller stages of mouse development (Theiler 1989) and Carnegie stages of human development (O'Rahilly and Müller 2010).

signals received from the fetus leading to an increased size of the nutrient exchange domains and an increased perfusion rate from the dam's circulation as a mechanism to prevent intrauterine growth restriction.

Alternatively, many $PM_{2.5}$ components contribute to oxidative stress that may have an impact on the function of the placenta. A recent study by Saenen et al. (2016) showed that exposure to a 7.5 $\mu g/m^3$ increase in $PM_{2.5}$ concentration during the second trimester in human pregnancies was associated with a 1.4% decrease in placental leptin gene methylation. Decreased methylation generally results in increased transcription of the methylated gene. Because leptin is a hormone involved in the proliferation and survival of placental trophoblast cells (Maymó et al. 2011), it may play a role in the alterations in placental weight observed in periods 3 and 4 (GD14.5–16.5 and GD0.5–16.5, respectively) in the present study. Additional studies are required to determine the potential role of leptin in this model.

Given that maternal exposure to $PM_{2.5}$ resulted in both PTB and LBW in this study, the observed subsequent lack of effects on postnatal growth rate was surprising. Human studies have shown that small-for-gestational-age size at birth is associated with increased risk of cardiovascular disease and type 2 diabetes in adulthood (Barker et al. 1989; Barker et al. 1990; Barker et al. 1993a, b; Phipps et al. 1993). *In utero* exposure to $PM_{2.5}$, which has independently been shown to predispose children to these same later-life outcomes (Johnson and Breslau 2000; Lewandowski et al. 2013; Li et al. 2014), could, in combination with small-for-gestational-age size, pose a synergistic increase in risk for these same obstetric consequences. A recent study by Janssen et al. (2016) showed a link between human exposure to an 8.2 $\mu g/m^3$ increase in $PM_{2.5}$ exposure levels in the third trimester and decreased thyroid stimulating hormone (TSH) levels and free thyroxine to triiodothyronine ratio (T_4/T_3) in cord blood. The decrease in free T_4 in cord blood was linked to a 56-g decrease in average birth weight. This finding differs from those in the present study, where exposure that occurred before the equivalent of the third trimester was associated with LBW. However, GD17.5 fetuses from dams exposed either throughout gestation or only during the third trimester analog were significantly lighter. Additional studies are warranted to determine the possible role for thyroid hormones in LBW due to $PM_{2.5}$ exposure.

In the present study, AGD in male offspring was reduced on PND10 and PND21 following maternal exposure to $PM_{2.5}$ throughout and early during gestation. In males, AGD is an indirect measure of total androgen exposure (both endogenous and exogenous) during fetal development; typically, the greater the exposure *in utero* to androgens, the greater the AGD (McIntyre et al. 2001). Shortening of the AGD, as was observed in this study following exposure during specific periods of development, has been used as an indicator of developmental exposure to antiandrogens such as phthalates (Foster 2006; Gray et al. 2006; Swan 2008). In humans, a shorter AGD in males has been linked to reductions in semen quality as defined by alterations in sperm morphology, motility, and total sperm per ejaculate (Mendiola et al. 2011; Swan et al. 2005). Interestingly, it has been observed in this laboratory (J.L.B. et al. 2013, unpublished work) that sperm numbers/motility were decreased in adult offspring in response to CAPs exposure throughout gestation at similar inhaled concentrations.

Increased AGD in females is also regulated by the secretion of androgens *in utero* (Wolf et al. 2002). In the present study, maternal exposure to CAPs early in and throughout pregnancy resulted in decreased AGD in females on PND10 that persisted through PND21. Although the underlying mechanism (or mechanisms) for such a finding is as yet unknown, AGD has been positively associated with the number of recruited ovarian follicles in women (Mendiola et al. 2012). In rat litters, female siblings with longer AGDs had greater pituitary responsiveness to gonadotropin releasing hormone than their sisters with shorter AGDs (Faber et al. 1993). The results from those studies suggest that changes associated with altered AGD in females brought about by *in utero* exposure to $PM_{2.5}$ may result in reduced fertility in the female offspring.

The present study has several limitations. In our study, the mice were exposed to higher $PM_{2.5}$ concentrations than would ordinarily be observed in human epidemiology studies, and it is not clear if the effects seen with short-term high-concentration exposures emulate those seen in constant, long-term exposures that could be experienced under conditions of pregnancy. However, when concentration is calculated based on the breathing rates of both species, the dose to the lung was only ~ 5 times greater in the mouse than in pregnant women. Additionally, $PM_{2.5}$ composition has been shown to vary from place to place with possible temporal variations within a single location. This study attempted to account for seasonal variation by performing experiments only in the summer and winter and to limit temporal effects by exposing the mice during all exposure periods at the same time so there would not be a bias in the event of a particularly high ambient air pollution day. Thus, although the confines of the study are recognized, the results of this animal study represent an important step forward in understanding the effects of maternal exposure to particulate air pollution across the gestational time span.

Conclusions

The study described herein presents biological feasibility for the epidemiologic studies demonstrating the adverse effects of inhaled particulate matter from air pollution on pregnancy-related outcomes. Moreover, these studies demonstrate the usefulness of a pregnant mouse model for studying the developmental consequences of exposure to $PM_{2.5}$. Such a model eliminates many confounding variables that often cloud human studies; it also provides the opportunity to confine exposures to a particular

gestational time period, making data interpretation easier. The results of this study also contribute to a better understanding of how and to what extent exposure periods play a role in predicting gestational outcomes.

Based on the findings here, exposure to $PM_{2.5}$ before implantation is not related to PTB, whereas maternal exposure postimplantation appears to pose a credible risk. In contrast, LBW appears to be linked with $PM_{2.5}$ exposure that occurs any time before the completion of embryogenesis. These animal studies suggest that exposure to high levels of inhaled $PM_{2.5}$ during pregnancy poses a risk for obstetric consequences during most gestational periods. Although it is difficult to avoid exposure to air pollution during pregnancy, certain interventions including the use of home air filters and air conditioners could help mitigate the risk for adverse pregnancy outcomes.

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References

Barker DJP, Bull AR, Osmond C, Simmonds SJ. 1990. Fetal and placental size and risk of hypertension in adult life. BMJ 301(6746):259–262, PMID: 2390618, https://doi.org/10.1136/bmj.301.6746.259.

Barker DJP, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. 1993a. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. Diabetologia 36(1):62–67, PMID: 8436255, https://doi.org/10.1007/bf00399095.

Barker DJP, Osmond C, Simmonds SJ, Wield GA. 1993b. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. BMJ 306(6875):422–426, PMID: 8461722, https://doi.org/10.1136/bmj.306.6875.422.

Barker DJP, Winter PD, Osmond C, Margetts B, Simmonds SJ. 1989. Weight in infancy and death from ischaemic heart disease. Lancet 2(8663):577–580, PMID: 2570282, https://doi.org/10.1016/S0140-6736(89)90710-1.

Basu R, Harris M, Sie L, Malig B, Broadwin R, Green R. 2014. Effects of fine particulate matter and its constituents on low birth weight among full-term infants in California. Environ Res 128:42–51, PMID: 24359709, https://doi.org/10.1016/j.envres.2013.10.008.

Bell ML, Belanger K, Ebisu K, Gent JF, Lee HJ, Koutrakis P, et al. 2010. Prenatal exposure to fine particulate matter and birth weight: variations by particulate constituents and sources. Epidemiology 21(6):884–891, PMID: 20811286, https://doi.org/10.1097/EDE.0b013e3181f2f405.

Blum JL, Xiong JQ, Hoffman C, Zelikoff JT. 2012. Cadmium associated with inhaled cadmium oxide nanoparticles impacts fetal and neonatal development and growth. Toxicol Sci 126(2):478–486, PMID: 22240978, https://doi.org/10.1093/toxsci/kfs008.

Chen LC, Hwang JS. 2005. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. IV. Characterization of acute and chronic effects of ambient air fine particulate matter exposures on heart-rate variability. Inhal Toxicol 17(4–5):209–216, PMID: 15804938, https://doi.org/10.1080/08958370590912789.

de Melo JO, Soto SF, Katayama IA, Wenceslau CF, Pires AG, Veras MM, et al. 2015. Inhalation of fine particulate matter during pregnancy increased IL-4 cytokine levels in the fetal portion of the placenta. Toxicol Lett 232(2):475–480, PMID: 25481569, https://doi.org/10.1016/j.toxlet.2014.12.001.

Ebisu K, Bell ML. 2012. Airborne $PM_{2.5}$ chemical components and low birth weight in the northeastern and mid-Atlantic regions of the United States. Environ Health Perspect 120(12):1746–1752, PMID: 23008268, https://doi.org/10.1289/ehp.1104763.

Faber KA, Ayyash L, Dixon S, Hughes CL Jr. 1993. Effect of neonatal diethylstilbestrol exposure on volume of the sexually dimorphic nucleus of the preoptic area of the hypothalamus and pituitary responsiveness to gonadotropin-releasing hormone in female rats of known anogenital distance at birth. Biol Reprod 48(5):947–951, PMID: 8481480, https://doi.org/10.1095/biolreprod48.5.947.

Foster PM. 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int J Androl 29(1):140–147, PMID: 16102138, https://doi.org/10.1111/j.1365-2605.2005.00563.x.

Gray LE Jr, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, et al. 2006. Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. Int J Androl 29(1):96–104, PMID: 16466529, https://doi.org/10.1111/j.1365-2605.2005.00636.x.

Ha S, Hu H, Roussos-Ross D, Haidong K, Roth J, Xu X. 2014. The effects of air pollution on adverse birth outcomes. Environ Res 134:198–204, PMID: 25173052, https://doi.org/10.1016/j.envres.2014.08.002.

Harris G, Thompson WD, Fitzgerald E, Wartenberg D. 2014. The association of PM (2.5) with full term low birth weight at different spatial scales. Environ Res 134:427–434, PMID: 25261950, https://doi.org/10.1016/j.envres.2014.05.034.

Huang RJ, Zhang Y, Bozzetti C, Ho KF, Cao JJ, Han Y, et al. 2014. High secondary aerosol contribution to particulate pollution during haze events in China. Nature 514(7521):218–222, PMID: 25231863, https://doi.org/10.1038/nature13774.

Huynh M, Woodruff TJ, Parker JD, Schoendorf KC. 2006. Relationships between air pollution and preterm birth in California. Paediatr Perinat Epidemiol 20(6):454–461, PMID: 17052280, https://doi.org/10.1111/j.1365-3016.2006.00759.x.

Janssen BG, Saenen ND, Roels HA, Madhloum N, Gyselaers W, Lefebvre W, et al. 2016. Fetal thyroid function, birth weight, and *in utero* exposure to fine particle air pollution: A birth cohort study. Environ Health Perspect 125(4):699–705, PMID: 27623605, https://doi.org/10.1289/EHP508.

Jiang LL, Zhang YH, Song GX, Chen GH, Chen BH, Zhao NQ, et al. 2007. A time series analysis of outdoor air pollution and preterm birth in Shanghai, China. Biomed Environ Sci 20(5):426–431, PMID: 18188997.

Johnson EO, Breslau N. 2000. Increased risk of learning disabilities in low birth weight boys at age 11 years. Biol Psychiatry 47(6):490–500, PMID: 10715355, https://doi.org/10.1016/S0006-3223(99)00223-1.

Lewandowski AJ, Augustine D, Lamata P, Davis EF, Lazdam M, Francis J, et al. 2013. Preterm heart in adult life: cardiovascular magnetic resonance reveals distinct differences in left ventricular mass, geometry, and function. Circulation 127(2):197–206, PMID: 23224059, https://doi.org/10.1161/CIRCULATIONAHA.112.126920.

Li S, Zhang M, Tian H, Liu Z, Yin X, Xi B. 2014. Preterm birth and risk of type 1 and type 2 diabetes: Systematic review and meta-analysis. Obes Rev 15(10):804–811, PMID: 25073871, https://doi.org/10.1111/obr.12214.

Maciejczyk P, Zhong M, Li Q, Xiong J, Nadziejko C, Chen LC. 2005. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. II. The design of a CAPs exposure system for biometric telemetry monitoring. Inhal Toxicol 17(4–5):189–197, PMID: 15804936, https://doi.org/10.1080/08958370590912743.

Malmqvist E, Rignell-Hydbom A, Tinnerberg H, Björk J, Stroh E, Jakobsson K, et al. 2011. Maternal exposure to air pollution and birth outcomes. Environ Health Perspect 119(4):553–558, PMID: 21212043, https://doi.org/10.1289/ehp.1002564.

March of Dimes. 2014. Premature Birth Report Card. 2014. http://www.marchofdimes.org/materials/premature-birth-report-card-united-states.pdf [accessed 11 February 2015].

Maymó JL, Pérez Pérez A, Gambino Y, Calvo JC, Sánchez-Margalet V, Varone CL. 2011. Review: Leptin gene expression in the placenta–regulation of a key hormone in trophoblast proliferation and survival. Placenta 32 (suppl 2):S146–S153, PMID: 21303721, https://doi.org/10.1016/j.placenta.2011.01.004.

McIntyre BS, Barlow NJ, Foster PM. 2001. Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci 62(2):236–249, PMID: 11452136, https://doi.org/10.1093/toxsci/62.2.236.

Mendiola J, Roca M, Mínguez-Alarcón L, Mira-Escolano MP, López-Espín JJ, Barrett ES, et al. 2012. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. Environ Health 11:90, PMID: 23217457, https://doi.org/10.1186/1476-069X-11-90.

Mendiola J, Stahlhut RW, Jørgensen N, Liu F, Swan SH. 2011. Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York. Environ Health Perspect 119(7):958–963, PMID: 21377950, https://doi.org/10.1289/ehp.1103421.

O'Connor AR, Fielder AR. 2007. Visual outcomes and perinatal adversity. Semin Fetal Neonatal Med 12(5):408–414, PMID: 17704016, https://doi.org/10.1016/j.siny.2007.07.001.

O'Rahilly R, Müller F. 2010. Developmental stages in human embryos: revised and new measurements. Cells Tissues Organs 192(2):73–84, PMID: 20185898, https://doi.org/10.1159/000289817.

Pereira G, Belanger K, Ebisu K, Bell ML. 2014. Fine particulate matter and risk of preterm birth in Connecticut in 2000-2006: A longitudinal study. Am J Epidemiol 179(1):67–74, PMID: 24068199, https://doi.org/10.1093/aje/kwt216.

Phipps K, Barker DJP, Hales CN, Fall CH, Osmond C, Clark PM. 1993. Fetal growth and impaired glucose tolerance in men and women. Diabetologia 36(30):225–228, PMID: 8462770, https://doi.org/10.1007/BF00399954.

Rappazzo KM, Daniels JL, Messer LC, Poole C, Lobdell DT. 2014. Exposure to fine particulate matter during pregnancy and risk of preterm birth among women in New Jersey, Ohio, and Pennsylvania, 2000–2005. Environ Health Perspect 122(9):992–997, PMID: 24879653, https://doi.org/10.1289/ehp.1307456.

Ritz B, Wilhelm M, Hoggatt KJ, Ghosh JK. 2007. Ambient air pollution and preterm birth in the environment and pregnancy outcomes study at the University of California, Los Angeles. Am J Epidemiol 166(9):1045–1052, PMID: 17675655, https://doi.org/10.1093/aje/kwm181.

Ritz B, Yu F, Chapa G, Fruin S. 2000. Effect of air pollution on preterm birth among children born in Southern California between 1989 and 1993. Epidemiology 11(5):502–511, PMID: 10955401, https://doi.org/10.1097/00001648-200009000-00004.

Saenen ND, Vrijens K, Janssen BG, Roels HA, Neven KY, Vanden Berghe W, et al. 2016. Lower placental leptin promoter methylation in association with fine particulate matter air pollution during pregnancy and placental nitrosative stress at birth in the ENVIRONAGE Cohort. Environ Health Perspect 125(2):262–268, PMID: 27623604, https://doi.org/10.1289/EHP38.

Samet JM, Dominici F, Zeger SL, Schwartz J, Dockery DW. 2000. The National Morbidity, Mortality, and Air Pollution Study. Part I: Methods and methodologic issues. Res Rep Health Eff Inst (94 Pt 1):5–14, PMID: 11098531.

Schwab JJ, Felton HD, Demerjian KL. 2004. Aerosol chemical composition in New York State from integrated filter samples: Urban/rural and seasonal contrasts. J Geophys Res 109(D6):1–13, https://doi.org/10.1029/2003JD004078.

Sioutas C, Kim S, Chang M. 1999. Development and evaluation of a prototype ultrafine particle concentrator. J Aerosol Sci 30(8):1001–1012, https://doi.org/10.1016/S0021-8502(98)00769-1.

Srám RJ, Binková B, Dejmek J, Bobak M. 2005. Ambient air pollution and pregnancy outcomes: a review of the literature. Environ Health Perspect 113(4):375–382, PMID: 15811825, https://doi.org/10.1289/ehp.6362.

Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 113(8):1056–1061, PMID: 16079079, https://doi.org/10.1289/ehp.8100.

Swan SH. 2008. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res 108(2):177–184, PMID: 18949837, https://doi.org/10.1016/j.envres.2008.08.007.

Symanski E, Davila M, McHugh MK, Waller DK, Zhang X, Lai D. 2014. Maternal exposure to fine particulate pollution during narrow gestational periods and newborn health in Harris County, Texas. Matern Child Health J 18(8):2003–2012, PMID: 24585411, https://doi.org/10.1007/s10995-014-1446-7.

Theiler K. 1989. *The House Mouse: Atlas of Embryonic Development*. New York, NY:Springer-Verlag 178 pp.

Thurston GD, Ito K, Lall R. 1994. A source apportionment of U.S. fine particulate matter air pollution. Atmos Environ (1994) 45(24):3924–3936, PMID: 24634604, https://doi.org/10.1016/j.atmosenv.2011.04.070.

U.S. Environmental Protection Agency (EPA). 2012. Responses to Significant Comments on the 2012 Proposed Rule on the National Ambient Air Quality Standards for Particulate Matter (June 29, 2012; 77 FR 38890). Docket Number EPA–HQ–OAR–2007–0492. https://www3.epa.gov/ttn/naaqs/standards/pm/data/20121214rtc.pdf [accessed 11 February 2015].

U.S. EPA. 2013. National Ambient Air Quality Standards for Particulate Matter; Final Rule. Fed Reg 78 (10):3086–3287. http://www.gpo.gov/fdsys/pkg/FR-2013-01-15/pdf/2012-30946.pdf [accessed 11 February 2015].

Veras MM, Damaceno-Rodrigues NR, Caldini EG, Maciel Ribeiro AA, Mayhew TM, Saldiva PH, et al. 2008. Particulate urban air pollution affects the functional morphology of mouse placenta. Biol Reprod 79(3):578–584, PMID: 18509159, https://doi.org/10.1095/biolreprod.108.069591.

Wolf CJ, Hotchkiss A, Ostby JS, LeBlanc GA, Gray LE Jr 2002. Effects of prenatal testosterone propionate on the sexual development of male and female rats: A dose-response study. Toxicol Sci 65(1):71–86, PMID: 11752687, https://doi.org/10.1093/toxsci/65.1.71.

Xu X, Ding H, Wang X. 1995. Acute effects of total suspended particles and sulfur dioxides on preterm delivery: a community-based cohort study. Arch Environ Health 50(6):407–415, PMID: 8572718, https://doi.org/10.1080/00039896.1995.9935976.

Yip FY, Pearcy JN, Garbe PL, Truman BI. Centers for Disease Control and Prevention (CDC), 2011. Unhealthy air quality - United States, 2006-2009. MMWR Suppl 60(1):28–32, PMID: 21430616.

Zhao Q, Liang Z, Tao S, Zhu J, Du Y. 2011. Effects of air pollution on neonatal prematurity in Guangzhou of China: a time-series study. Environ Health 10:2, PMID: 21214958, https://doi.org/10.1186/1476-069X-10-2.